



UNIVERSITI PUTRA MALAYSIA

**WNT SIGNALING PATHWAY RELATED GENES IN
HEPATOCELLULAR CARCINOMA**

KECHEN BAN

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**WNT SIGNALING PATHWAY RELATED GENES IN
HEPATOCELLULAR CARCINOMA**

**By
KECHEN BAN**

**Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfilment of the Requirement for the Degree of Doctor of Philosophy**

October 2002



Specially dedicated to,

My beloved grandmother, mother, aunts, brother, sisters, wife and son.

The memory of

My late grandfather, father and uncle.

For their invaluable love, understanding, tolerance, sacrifice and moral support.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of
the requirement for the degree of Doctor of Philosophy

**WNT SIGNALING PATHWAY RELATED GENES IN
HEPATOCELLULAR CARCINOMA**

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October 2002

Chairman: Associate Professor Dr. Seow Heng Fong

Faculty: Medicine and Health Sciences

Hepatocellular carcinoma (HCC) is an extremely difficult disease to manage and one of the ten most common cancers in Malaysia. However, the molecular mechanisms that contribute to tumour progression in hepatocarcinogenesis remain unknown.

In recent years, the evidence that the Wnt signaling pathway may play a crucial role in the hepatocarcinogenesis represents a major breakthrough. Despite efforts made in this area of research, the relationship between the related genes of Wnt signaling pathway is still far from comprehensive.

By employing immunohistochemistry and DNA sequencing, some related proteins and genes of the Wnt signaling pathway were investigated in 23 paired HCC and adjacent liver specimens from a tertiary referral centre in Malaysia.

The results showed that:

1. The overexpression of p53, β -catenin, Akt, phosphorylated glycogen synthase kinase (phospho-GSK)-3 β , cyclin D1, cyclooxygenase(COX)-2 and Wnt-1 was found in 26.1%, 56.5%, 52.2%, 52.2%, 52.2%, 26.1% and 8.7% of HCC tissues, respectively, and 0, 39.1%, 65.2%, 26.1%, 4.3%, 47.8%, 0 in non-cancerous surrounding tissues, respectively. The expression of β -catenin, phospho-GSK-3 β and cyclin D1 in HCC tissues was higher than in surrounding tissues, respectively (all $p < 0.05$). These results suggest that p53, β -catenin, phospho-GSK-3 β and cyclin D1 may play a role in HCC from Malaysia.
2. In non-cancerous liver tissues, there was a possible relationship between the overexpression of COX-2 and β -catenin, phospho-GSK-3 β and β -catenin, Akt and phospho-GSK-3 β , respectively (all $p < 0.05$).
3. In HCC tissues, there was a possible relationship between the expression of phospho-GSK-3 β and p53, cyclin D1 and p53, β -catenin and p53, respectively (all $p < 0.05$).
4. According to above findings, it is likely that the development of HCC involves the Akt-GSK-3 β , Akt-GSK-3 β -cyclin D1 and COX-2- β -catenin pathways which was mediated or not mediated by p53. In addition, our results also supported that Wnt signaling pathway was involved in hepatocarcinogenesis.
5. The results of nucleotide sequencing showed that no mutations at codon 249 of p53 and at the four potential consensus GSK-3 β phosphorylation sites of β -catenin were

found. Thus, it is likely that the increased expression of β -catenin resulted from the overexpression of phospho-GSK-3 β and COX-2.

This study is the first one to demonstrate that the related genes of the Wnt signaling pathway are involved in the hepatocarcinogenesis in Malaysia

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

GEN-GEN YANG BERKAITAN DENGAN LINTASAN ISYARAT “WNT” PADA BARAH HATI

Oleh

KECHEN BAN

Oktober 2002

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Barah hati merupakan satu penyakit yang amat sukar diubati dan adalah salah satu dari sepuluh barah yang sering dihidapi umum di Malaysia. Walaubagaimanapun, mekanisma-mekanisma molekul yang menyumbang kepada pertumbuhan barah hati masih belum diketahui.

Dikebelakangan ini, bukti yang menunjukkan lintasan isyarat “Wnt” memainkan peranan dalam pertumbuhan barah hati telah menjadi satu penemuan yang mustahak. Walaupun banyak usaha telah diambil, hubungan antara gen-gen yang berkaitan dengan lintasan isyarat “Wnt” masih tidak jelas.

Beberapa protein dan gen yang berkaitan dengan lintasan isyarat “Wnt” telah dikaji di dalam 23 pasang spesimen tisu barah hati dan tisu sekeliling dari pusat rujukan tertier Malaysia dengan penggunaan immunohistokimia dan penjujukan DNA.

Keputusan-keputusan yang diperolehi menunjukkan bahawa:

1. Pengekspresan berlebihan bagi p53, β -catenin, Akt, glikogen synthase kinase (fosfo-GSK)-3 β , cyclin D1, cyclooxygenase (COX)-2 dan Wnt-1 yang didapati adalah sebanyak 26.1%, 56.5%, 52.2%, 52.2%, 52.2%, 26.1% dan 8.1% masing-masing di tisu barah hati berbanding dengan 0, 39.1%, 65.2%, 26.1%, 4.3%, 47.8% dan 0 di tisu sekeliling. Pengekspresan β -catenin, fosfo-GSK-3 β dan cyclin D1 di dalam tisu barah hati adalah lebih tinggi berbanding dengan tisu sekeliling ($p < 0.05$). Keputusan ini mencadangkan bahawa p53, β -catenin, fosfo-GSK-3 β dan cyclin D1 memainkan peranan dalam barah hati di Malaysia.
2. Di dalam tisu hati yang bukan barah, hubungan pengekspresan yang berlebihan wujud bagi pasangan gen-gen yang berikut: COX-2 dan β -catenin, fosfo-GSK-3 β dan β -catenin serta Akt dan fosfo-GSK-3 β ($p < 0.05$).
3. Di dalam tisu barah hati pula, hubungan pengekspresan yang berlebihan wujud bagi pasangan gen-gen yang berikut: fosfo-GSK-3 β dan p53, cyclin D1 dan p53 serta β -catenin dan p53.
4. Penemuan di atas mekadangkan bahawa pertumbuhan barah hati melibatkan lintasan Akt-GSK-3 β , Akt-GSK-3 β -cyclin D1 dan COX-2- β -catenin yang samada dipandu oleh p53. Selain itu, keputusan yang diperolehi juga menyokong penglibatan lintasan isyarat "Wnt" dalam proses pertumbuhan barah hati.

5. Keputusan penjujukan menunjukkan bahawa tiada mutasi yang berlaku pada kodon 249 bagi p53 serta empat lokasi yang berpotensi bagi pemfosforilasi GSK-3 β . Oleh itu, peningkatan pengespresan β -catenin kemungkinan besar disebabkan oleh pengekspresan fosfo-GSK-3 β dan COX-2 yang berlebihan.

Kajian ini merupakan kajian yang pertama menunjukkan penglibatan gen-gen yang berkaitan dengan lintasan isyarat “Wnt” dalam pertumbuhan barah hati di Malaysia.

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I certify that an Examination Committee met on 22nd October 2002 to conduct the final examination of Kechen Ban on his Doctor of Philosophy thesis entitled "Wnt Signaling Pathway Related Genes in Hepatocellular Carcinoma" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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LIST OF ABBREVIATIONS

AFB1	Aflatoxin B1
AFP	Alpha-foetoprotein
APC	Adenomatous polyposis coli
APES	3-Aminopropyltrimethoxysilane
bp	Base pair
°C	Celsius degree
CaCl ₂	Calcium chloride
cAMP	Cyclic adenosine monphosphate
CDK	Cyclin-dependent kinases
COX	Cyclooxygenase
DAB	3,3'-Diaminobenzidine
dH ₂ O	Distilled water
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTPs	Dideoxynucleotide triphosphates (dATP, dTTP, dCTP and dGTP)
EDTA	ethylenediaminetetraacetic acid
Fz	Frizzled
g	Gram
GSK	Glycogen synthase kinase
h	Hour(s)
HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid
HCV	Hepatitis C virus
IFN-γ	Interferon-gamma
LCM	Laser capture microdissection
LOH	Loss of heterozygosity
kb	Kilobase pair
mA	Milliamperes
mg	Milligram
MgCl ₂	Magnesium chloride
min(s)	Minute(s)
ml	Milliliter
mM	Millimolar
mRNA	Message ribonucleic acid
n	Nano
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ORF	Open reading frame
PBS	Phosphate buffered saline
PCI	Phenol/chloroform/isoamyl alcohol
PCR	Polymerase chain reaction
Phospho-GSK	Phosphorylated glycogen synthase kinase



PKB	Protein kinase B
RNA	Ribonucleic acid
rpm	Revolutions per minute
s	Second(s)
TAE	Tris acetate EDTA buffer
Taq	Thermus aquaticus thermostable DNA
Tcf/LEF	T cell factor/lymphoid enhancer factor
μl	Microlitre
μg	Microgram
v/v	Volume per unit volume
WHO	World health organization

CHAPTER I

INTRODUCTION

Hepatocellular carcinoma (HCC) occurs at high frequencies in East Asia and sub-Saharan Africa, representing 4% of all malignant tumours, and is the seventh most frequent carcinoma in males and the ninth most frequent carcinoma in females (Buendia *et al.*, 1992; Ng *et al.*, 1994; Caselmann *et al.*, 1996). It has been estimated that there are 1 million cases per year in the world. The major aetiologies of HCC include chronic infection with the hepatitis B virus (HBV) and hepatitis C virus (HCV) and exposure to the fungal toxin aflatoxin B1 (AFB1) (Hussain *et al.*, 2001). The rates of HBV infection varies widely. In Nigeria, Benin and China including Taiwan, the incidence is high, while in the continental United States and in western Europe the incidence is relatively low. On the contrary, the prevalence of HCV antibody-positive patients with HCC was relatively higher in Japan, South Africa, Europe and USA (Edamoto *et al.*, 1996).

In Malaysia, about 5% of healthy volunteers were positive for hepatitis B surface antigen (HBsAg) in 1997 (Merican *et al.*, 2000) and HCC is one of the ten most common cancers amongst the male population in this country (Merican, 1996).

HCC is an extremely difficult disease to manage. The treatment modalities for HCC have made little progress in the past ten years. Moreover, recent reports showed that the incidence of HCC is increasing in low to middle-incidence countries such as the USA, France, UK and most particularly, Japan (Anthony *et al.*, 2001). Therefore, it is

important to elucidate the molecular mechanisms of hepatocarcinogenesis which remains unknown.

It is believed that HCC is a disease of multiple modalities. So far, many signaling pathways have been implicated in hepatocarcinogenesis.

In recent years, the evidence that the Wnt signaling pathway plays a crucial role in the hepatocarcinogenesis represents a major breakthrough (Buendia *et al.*, 2000). Intracellular protein complexes, including Dishevelled (Dvl/Dsh), glycogen synthase kinase-3 β (GSK-3 β), axin and adenomatous polyposis coli (APC) protein, regulate cytosolic β -catenin protein levels.

The β -catenin, like its homologue armadillo in *Drosophila*, is an important multifunctional protein involved in cell-cell adhesion, by strengthening the linkage of cadherin and α -catenin to the actin cytoskeleton (Aberle *et al.*, 1996). It is also involved in Wnt signaling during embryonic development (Cadigan *et al.*, 1997) and inappropriate reactivation of this pathway has been implicated in tumourigenesis. In the absence of Wnt signaling, β -catenin is phosphorylated at N-terminal serine-threonine residues by functional interactions with GSK-3 β , axin and APC, and subsequently targeted to degradation by the ubiquitin-proteasome system (Polakis *et al.*, 1997). Activation of the Wnt signal inhibits GSK-3 β activity and induces β -catenin stabilization. Translocation of β -catenin to the nucleus and its association with high mobility group domain factors T cell factor/lymphoid enhancer factor (Tcf/LEF) causes transcriptional activation of target genes (Clevers *et al.*, 1997). Recently, mutant β -

catenins that are resistant to down-regulation by GSK-3 β phosphorylation and ubiquitination have been characterized in a variety of carcinomas (Morin *et al.*, 1997, Iwao *et al.*, 1998; Polakis *et al.*, 1999). In colon cancers, immunohistochemical studies have demonstrated increased expression of β -catenin and its nuclear localization in tumours harboring either APC defects or β -catenin mutations in the GSK-3 β phosphorylation domain (Iwao *et al.*, 1998; Inomata *et al.*, 1996). Nuclear and cytoplasmic localization of β -catenin was also frequently seen in ovarian, uterine carcinomas and melanomas, although genetic alterations of the β -catenin gene could be detected only in a minority of these tumours (Palacios *et al.*, 1998; Fukuchi *et al.*, 1998; Rimm *et al.*, 1999).

In human HCC, mutations of the β -catenin gene have been reported in 19 to 41% of primary tumours (de La Coste *et al.*, 1998; Huang *et al.*, 1999). There were reports that the overexpression of β -catenin may be related to the proliferative activity and dedifferentiation of HCC (An *et al.*, 2001) and activation of the Wnt signaling pathway by β -catenin mutation contributes significantly to the hepatocellular carcinogenesis (Huang *et al.*, 1999).

Until June of 2002, the list of target genes of Wnt signaling covers 46 members (<http://www.stanford.edu/~musse/pathways/targets.html>), including cyclin D1 (Tetsu *et al.*, 1999; Shtutman *et al.*, 1999) and Cyclooxygenase(COX)-2 (Howe *et al.*, 1999; Haertel-Wiesmann *et al.*, 2000).

Both cyclin D1 and COX-2 are believed to play a role in hepatocarcinogenesis.

Cyclin D1 has been suggested to be rate-limiting for the progression from G₁ into S phase of the cell cycle, based on the observation that their constitutive overexpression leads to a shorter G₁ phase (Ando *et al.*, 1993; Musgrove *et al.*, 1994). Constitutive overexpression of cyclin D1 in mouse mammary glands leads to carcinoma, indicating that cyclin D1 acts as an oncogene (Wang *et al.*, 1994). Cyclin D1 have also been implicated in the development of HCC (Deane *et al.*, 2001; Nishida *et al.*, 1994).

Expression of COX-2 has been reported to be responsible for enhanced tumour growth and angiogenesis in various tumours (Sano *et al.*, 1995; Ristimaki *et al.*, 1997; Zimmermann *et al.*, 1999; Hida *et al.*, 1998), including HCC (Bae *et al.*, 2001; Rahman *et al.*, 2001; Kondo *et al.*, 1999; Shiota *et al.*, 1999; Koga *et al.*, 1999).

In recent years, some HCC-related genes have also been reported to be associated with the components or target genes of Wnt signaling pathway.

Abnormalities of p53 are the most frequent genetic alterations in human cancers (Hollstein *et al.*, 1991). The p53 tumour suppressor gene plays a key role in human carcinogenesis through regulating its target genes involved in cell cycle control and apoptosis in response to cellular damage (Levine *et al.*, 1997). The p53 gene has also been shown to play an important role in hepatocarcinogenesis. p53 mutations have been found in about 25–45% of HCCs (Montesano *et al.*, 1997). A unique mutation hotspot, a G to T transversion at the third base of codon 249 of the p53 gene was observed in the HCC tissues in the region with presumed high AFB1 exposure (Wang *et al.*, 1999). The presence of p53 mutations was associated with a shortened (cancer-free) survival of